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How do Combinations of Nutrients Cause Symbiotic *Chlorella* to Overgrow Hydra?

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Abstract

Population density of *Chlorella* sp., symbiotic with green hydra (Florida and European strains), is normally maintained at a characteristic level. As the host grows, the algae also grow, by asexual production of daughter cells (autospores). When these hydra are maintained in light and starved, and their normal maintenance medium is supplemented with a combination of inorganic nutrients, the population density of *Chlorella* increases substantially, and often leads to disintegration of the host after 6 to 9 days. The nutrient-supplemented solution also causes an increase in the frequency of cell division, and the frequency of production of eight autospores per cell. Preliminary tests using 27 different combinations of nutrients failed to reveal but did not rule out any ion-specific effect.

Keywords: symbiosis, hydra, nutrients, algae

1. Introduction

There is substantial evidence that the population size of *Chlorella* sp., living in symbiosis with *Hydra* sp. is regulated (see review of McAuley, 1985a). The evidence is based on the tendency for symbiotic algae and host digestive cells to maintain a stable biomass ratio (e.g. number of algae per digestive cell of the host) under a given set of culture conditions, or to achieve

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a new stable ratio when culture conditions are altered. The numbers of algae per host cell usually increase as a result of changing the daily irradiance regime from periodic to continuous, decreasing the temperature, starving the host, adding sulfate to the culture medium, or increasing its K^+/Na^+ ratio (McAuley, 1985a). The host cell can potentially prevent algal overgrowth by expelling or digesting algae in excess of the carrying capacity, or by inhibiting growth of the algae (Muscatine and Pool, 1979).

Inhibition of growth (i.e. cell division) of symbiotic *Chlorella* sp. could be effected in several ways, not necessarily mutually exclusive. One way is to lower the pH in the vacuoles containing the algae. Low pH stimulates release of maltose from the algae (Muscatine, 1965; Muscatine et al., 1967; Mews, 1980), thus diverting fixed carbon from growth (Douglas and Smith, 1984). Growth of algae might also be regulated by a factor which limits cell division. Thus, when hydra are fed *Artemia* nauplii previously extracted with an aqueous solvent, the growth rate of the algae but not the host cells decreases (McAuley, 1985b). Pertinent to the same regulatory principle, Pool (1976; see also Muscatine and Pool, 1979) found that when sulfate was added to hydra culture medium, the number of algae per cell increased, suggesting that this divalent inorganic ion, known to be required for completion of the cell cycle of free-living *Chlorella* sp. (Tamiya, 1963), was limiting the growth rate of symbiotic *Chlorella in situ*, and that its supply might be regulated by the host.

The potential role of inorganic nutrients in regulating the number of algae per cell in *Hydra* was investigated further by Muscatine and Neckelmann (1981), Neckelmann (1982) and Neckelmann and Muscatine (1983). They confirmed that sulfate added to the culture medium caused an increase in numbers of algae per digestive cell. In addition they demonstrated that when a combination of nutrients (including Fe^{2+}, NH_4^+, NO_3^-, PO_4^{3-}, SO_4^{2-}) was added to the culture medium, the mean number of algae per digestive cell increased from the usual 17 to an unprecedented 45 to 50, and after 8 to 10 days, some of the treated hydra disintegrated. Since aposymbiotic hydra continued to grow, although somewhat more slowly, in nutrient-supplemented medium, the disintegration of the host seemed more likely to be a secondary effect resulting from overgrowth by algae rather than from a primary effect of the altered medium on the animal cells. Finally, the overgrowth was due to some effect correlated with the addition of nutrients in combination, rather than strictly to the presence of sulfate, since combinations of nutrients lacking sulfate also evoked increased numbers of algae per cell.

The purpose of the present study was to gain further insight into the mechanism by which combinations of nutrients lead to increased numbers of algae per host cell. In this paper we demonstrate that nutrient-supplemented solutions affect the frequency and pattern of autospore production by symbiotic *Chlorella*.

2. Materials and Methods

Maintenance of hydra

Stock cultures of *Hydra viridis* (Florida and European strains) were maintained in glass trays in M solution, pH 7.4, (Muscatine and Lenhoff, 1965) in a Precision incubator at 18°C on a 12 hr light:12 hr dark cycle. Maintenance irradiance was 20 μE m^{-2} s^{-1} provided by two 40-watt fluorescent tubes (Sylvania Cool White). Hydra were fed three times weekly on freshly hatched *Artemia* larvae.

Supplementation of M solution with inorganic nutrients

M solution, pH 7.4, was supplemented with $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$, $Na_2EDTA{\cdot}2H_2O$ and KNO_3, each to a final concentration of 10^{-4}M, and KH_2PO_4 and Na_2SO_4, each to a final concentration of 10^{-3}M. The supplemented solution, adjusted to pH 7.4, is referred to as M+N.

Design of nutrient supplementation experiments and sampling protocol

All experiments were started on stock hydra which had not been fed for 24 hr. Hydra were transferred from stock solutions to plastic Petri dishes containing 100 ml test solution. Two replicate sets of dishes were prepared, each with 30 stock hydra per dish. These were maintained in an incubator at 18°C on either a 12 hr light:12 hr dark photoperiod at 20 μE $m^{-2}s^{-1}$ or under constant irradiance at 100 μE $m^{-2}s^{-1}$. The culture medium was replaced daily.

From one set of dishes, three hydra were sampled daily for up to 10 days and macerated to determine the number of algae per host digestive cell. Hydra were macerated by the method of Pardy and Muscatine (1973). For each maceration three individuals were treated with a drop of maceration fluid (water, glycerol, glacial acetic acid; 13:1:1), teased apart with needles, and examined with a Leitz Ortholux photomicroscope using phase contrast optics at 400×. The number of algae per digestive cell was determined from observation of 30 digestive cells randomly selected from each hydra.

From replicate sets of experimental hydra, three hydra were sampled daily for 5 days, homogenized in a 0.5 cm^3 glass micro-tissue grinder in a small volume of culture medium. The homogenate was centrifuged at a speed sufficient to pellet the algae. The supernatant was discarded and the algae transferred to a glass slide and examined at 1000× with an Olympus BHTU compound microscope using phase contrast optics. Five hundred algae were observed at random, the number of algal cells undergoing cytokinesis was determined, and the number of daughter cells produced by each division at the time of observation was recorded.

Electron microscopy

Hydra were fixed for electron microscopy as described by Muscatine and McAuley (1982). After dehydration in an ethanol series, specimens were treated for scanning (SEM) or transmission electron microscopy (TEM).

For SEM, specimens were subjected to ethanol cryofracture (Hymphreys et al., 1974). While still immersed in absolute ethanol, specimens were inserted into Parafilm tubes and frozen in liquid nitrogen. The frozen preparation was fractured in a single plane with a blow from a cold razor blade. The fractured pieces were immediately placed in absolute ethanol and critical-point dried in a Samdri PVT-3 apparatus. Specimens were glued to stubs, coated with palladium-gold (Technic Hummer 1 sputter coater) and scanned with an ETEC Autoscan microscope.

For TEM, after dehydration with propylene oxide, specimens were flat embedded in Medcast soft block mixture (Ted Pella 18009 one-mix system) and cut on a LKB Ultratome. Sections were mounted on grids coated with formvar and carbon, and were observed with a Philips 300 electron microscope either unstained or stained with 2% aqueous uranyl acetate.

3. Results

Effect of nutrient supplementation and irradiance on number of algae per host digestive cell

Muscatine and Neckelmann (1981) and Neckelmann and Muscatine (1983) established that stimulation of growth of algae by nutrient supplementation occurs principally when hydra are starved in light during the treatment period. Starvation also avoids the uncontrolled introduction of inorganic nutrients in *Artemia*. Using these conditions as a point of departure, we initially sought to demonstrate the effect of nutrient supplementation on numbers of algae per digestive cell in Florida and European hydra. Controls in M solu-

tion and nutrient-supplemented Florida and European hydra were incubated at 20 μE m^{-2} s^{-1} at 18°C on a 12 hr light: 12 hr dark photoperiod for 10 days without feeding. Hydra were sampled and macerated daily, and the number of algae per digestive cell ascertained.

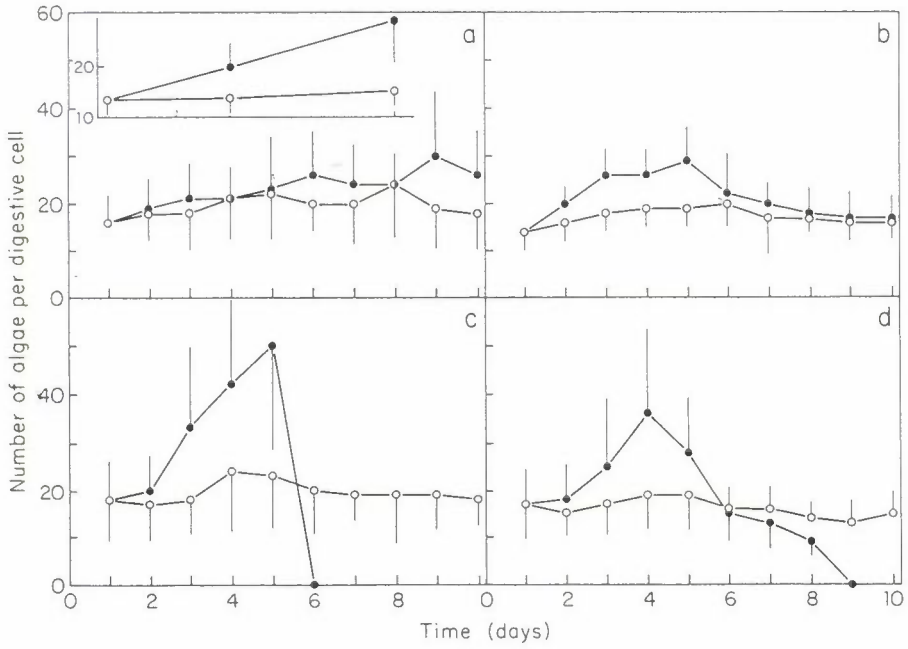

Figure 1. Number of algae per digestive cell in Florida and European hydra starved for 8 to 10 days in control M solution (o) and nutrient-supplemented M+N (•). (a) Florida, 20 μE m^{-2} s^{-1}, 12 hr light: 12 hr dark; (b) European, as in (a); (c) Florida, 100 μE $m^{-2}s^{-1}$, 24 hr constant light; (d) European, as in (c). Vertical lines denote standard error of the mean (n = 30).

Figure 1a (inset) shows that in a survey experiment, controls in M showed little or no change in number of algae per digestive cell, while those in M+N increased to about 30 per cell, or approximately double the control value, in 8 days. In a second trial (Fig. 1a), with daily sampling, the mean number of algae per cell in Florida hydra controls increased during the first 5 days of treatment and then fluctuated between 18 and 24 algae per cell. In hydra in M+N, the mean number per cell also increased, reaching 26 to 30 algae per cell after 10 days. Results with European hydra were similar (Fig. 1b), except that after 5 days in M+N, the number of algae per digestive cell decreased again to control levels.

Although the treatment with M+N led to a noticeable increase in number of algae per cell in Florida hydra, the increase was not as striking as that reported by Muscatine and Neckelmann (1981) and Neckelmann and Muscatine (1983). However, by changing to constant light and increasing the incubation irradiance to 100 μE $m^{-2}s^{-1}$, the stimulatory effect of M+N was revealed unequivocally. Fig. 1c shows that the number of algae per cell in Florida hydra increased dramatically over the first 5 days to more than 50, followed by immediate and rapid disintegration of the hydra. European hydra behaved similarly (Fig. 1d). The number of algae per cell increased to 36 during the first 4 days, then decreased for the next 5 days until all of the hydra eventually disintegrated.

Effect of nutrient supplementation on mitotic index and pattern of autospore production

To further characterize the effect of nutrient supplementation, we measured mitotic index and described the pattern of autospore production of algae from hydra sampled in the experiments above (Table 1).

Table 1. Mitotic index and percentage of *Chlorella* with two, four, and eight autospores in Florida hydra after 6 days starvation at 18°C, 20μE.m^{-2}.s^{-1}, 12 hr light:12 hr dark

	Control				M+N			
	Mitotic index	Autospores			Mitotic index	Autospores		
Day	(%)	2	4	8	(%)	2	4	8
0	1.5	0.8	0.7	0.0	1.5	0.8	0.7	0.0
1	1.6	0.9	0.7	0.0	2.2	1.2	0.9	0.1
2	1.1	0.7	0.4	0.0	4.0	2.0	1.7	0.3
3	1.6	1.0	0.5	0.1	4.1	1.9	1.9	0.3
4	1.7	1.4	0.3	0.0	4.2	1.8	2.0	0.4
5	2.1	1.5	0.4	0.2	3.2	1.4	1.4	0.4

In controls, mitotic index ranged from 1.1 to 2.1%, showing little increase over the 6 day starvation period. Two-cell and four-cell division stages predominated, with an indication that the percent of the former increased and the later decreased as starvation progressed. There was no evidence of eight-cell stage cells in algae from stock hydra, but as starvation progressed through days 4 and 6, about 10% of those cells in division were producing more than 4 autospores.

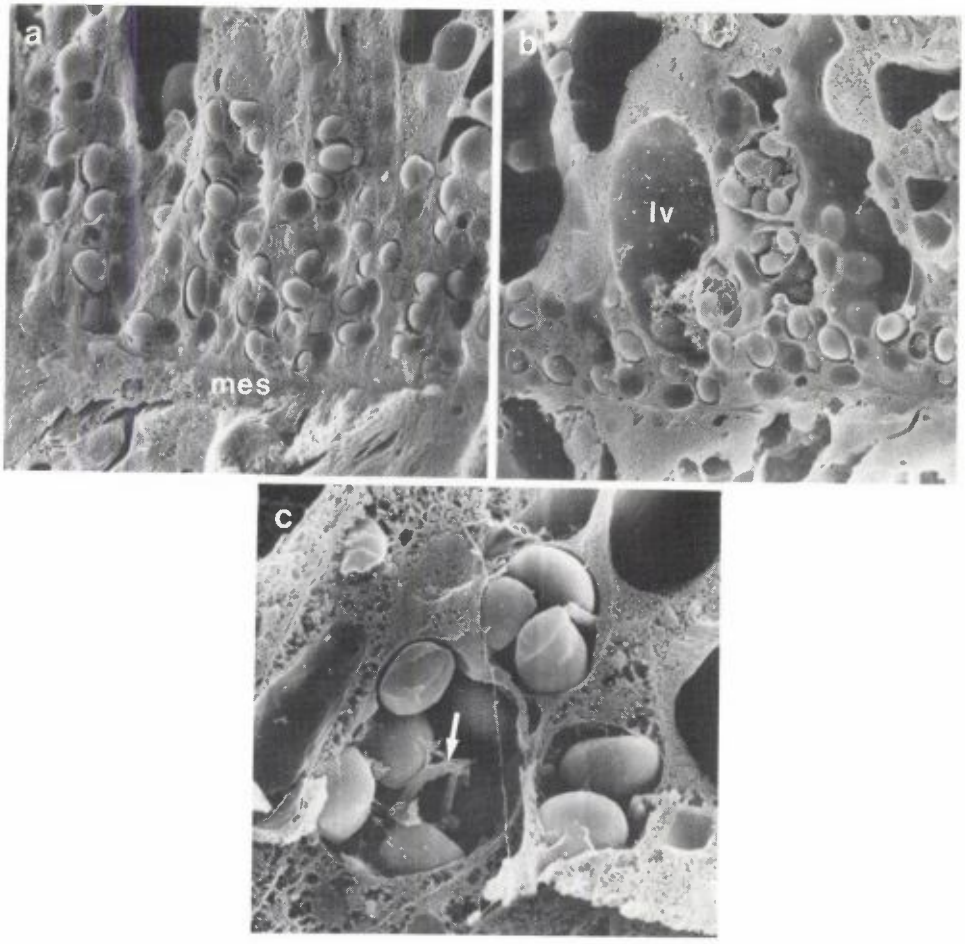

Figure 2. (a) SEM of cryofractured Florida hydra controls in M solution starved for 5 days, $100\mu E.m^{-2}.s^{-1}$ 1560×. (b) as above in M+N. 1560×. (c), as in (b), showing at least six daughter cells per vacuole and scrolled mother cell envelope. 4680×. lv, large vacuoles; mes, mesoglea; scrolled mother cell envelope at arrow.

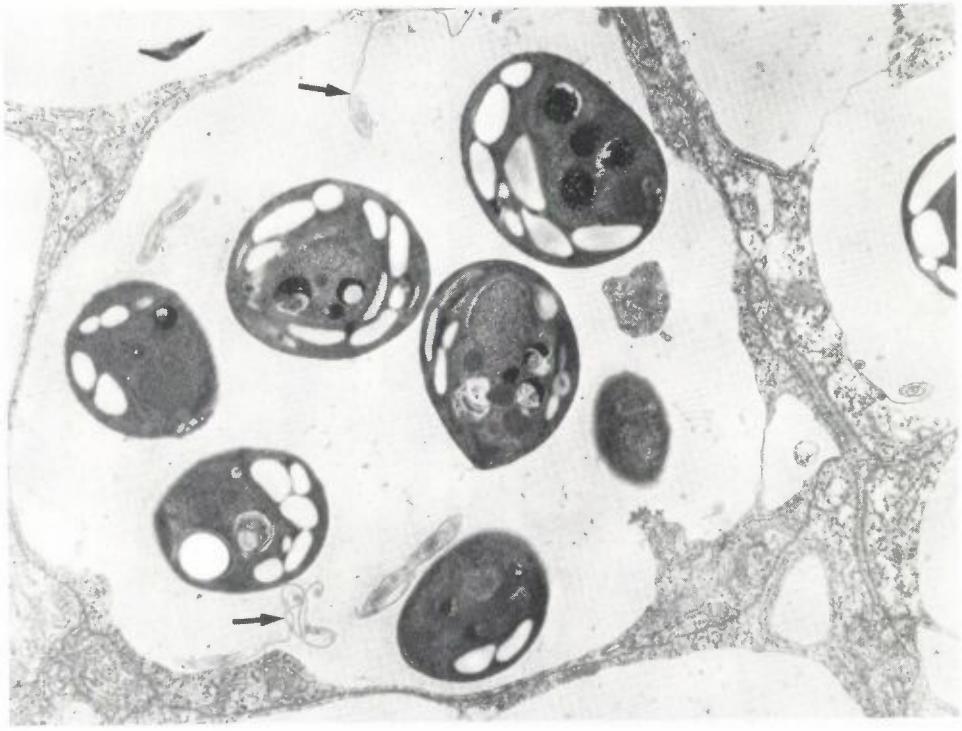

Figure 3. TEM of Florida hydra treated as in Fig. 2b showing seven of eight autospores in a single vacuole including mother cell envelope scrolls (at arrows). 10740×.

In nutrient supplemented hydra, mitotic index increased from 1.5 to 4.2% during the incubation period. There was some increase in the percentage of two-cell and four-cell stages, and a significant progressive increase in the proportion and time of appearance of eight-cell stages. The latter comprised an unprecedented 13% of the cells in division after 6 days starvation.

Effect of nutrient supplementation on morphology and ultrastructure of hydra

Control and experimental hydra were examined by SEM after cryofracture, and by TEM. The results of SEM are shown in Fig. 2. Cells in control hydra (Fig. 2a) are elongate, and the algae reside individually in close-fitting vacuoles. Other large vacuoles can be seen at the host cell apex. Cells in treated hydra are also elongate, and algae also appear individually in basal vacuoles, but elsewhere more than one alga per vacuole can be observed (Fig. 2b). Larger subsidiary vacuoles are evident throughout the cells. At least one presumed eight-cell stage can be seen in a vacuole, together with scrolled remnants of the mother cell envelope (Fig. 2b,c). TEM of such a vacuole, showing seven daughter cells in the plane of section and scrolled mother cell envelope is shown in Fig. 3.

4. Discussion

The results of this study show that when Florida and European hydra are starved in light in M solution supplemented with a combination of inorganic nutrients, the number of algae per host digestive cell increases and the host disintegrates within 6 or 9 days, respectively. Apparently, the factors which can potentially regulate numbers of algae per cell (expulsion, digestion, inhibition of cell division) are unable to do so under these conditions. The results confirm the previous observations of Muscatine and Neckelmann (1981) and Neckelmann and Muscatine (1983) and extend them to include European hydra.

Merely starving hydra in the light (Pardy and Muscatine, 1973, Fig. 1a, this study) can evoke an increase in number of algae per digestive cell in Florida hydra, but the increase is transient, and in a few days a new stable population level is reached.

Whereas Muscatine and Neckelmann (1981) and Neckelmann and Muscatine (1983) consistently observed overgrowth of hydra at 20 μE m^{-2} s^{-1} on a 12 hr light:12 hr dark photoperiod, we obtained only modest increases in numbers of alga per host digestive cell under these conditions. Only when irradiance was increased to 100 μE $m^{-2}s^{-1}$ did we evoke overgrowth of the host by the algae. We have no explanation for this difference in results, but since algae *in situ* in Florida hydra saturate at 125 to 200 μE $m^{-2}s^{-1}$(Phipps and Pardy, 1982; G. Muller-Parker, unpublished), the higher irradiance can be expected to support growth of symbiotic *Chlorella* at higher rates.

Symbiotic *Chlorella* sp. are thought to customarily produce four autospores (Oschman, 1967). Recently, McAuley and Muscatine (1986) mea-

sured the frequency of appearance of cells producing more than four autospores in control and starved European hydra. About 8% of the algae in division in controls exhibited more than four autospores. After 8 days starvation, the frequency increased to 13%. Similar results were obtained in the present study with Florida hydra. The frequency of eight-cell stages increased to 10% after 5 days starvation and to 13% after treatment with M+N. Donnan et al. (1985) suggest that the number of autospores produced by free-living *Chlorella fusca* and *Chlamydomonas reinhardtii* is correlated with cell size. However, McAuley and Muscatine (1986) found that after starvation of European hydra for up to 22 days, although the mean volume of algae which produced eight autospores was larger than that of algae which produced four autospores, the difference was not statistically significant, suggesting that the number of autospores was determined by other factors.

The added production of eight autospores could account for the rapid increase in the number of algae in experimental hydra. We interpret the subsequent disintegration of the hydra as the result of overgrowth by the algae. In Florida hydra, disintegration occurs somewhat precipitously (Fig. 1c). In European hydra (Fig. 1d), neither a 13% frequency of eight-cell division stages nor treatment with M+N causes such a precipitous disintegration. This could be due to the predisposition of European hydra digestive cells to expel or digest excess algae, a phenomen termed "sorting" by McAuley and Smith (1982).

The way in which nutrients in combination cause stable population regulation to be breached in hydra starved in light is not yet understood. Muscatine and Neckelmann (1981) stimulated overgrowth in Florida hydra by supplementing M solution with a combination of inorganic nutrients, or with addition of sulfate alone. Neckelmann (1982) caused numbers of algae per digestive cell to double by supplementing M solution with either the combination of phosphate, nitrate and sulfate, or ferrous ammonium sulfate and EDTA. In preliminary experiments, we tested all of these ions, individually and in 27 different combinations and obtained inconclusive results. A given ion could be stimulatory in one combination or even toxic in another combination. However, the element most frequently present when overgrowth was produced was iron. Since sulfate is known to be a carrier for iron (A. Lewis, per. comm.), our attempts to systematically pinpoint a specific ion or combination of ions may have been thwarted by the incidence of such carrier phenomena and by the inclusion of trace ions which normally contaminate inorganic chemicals.

McAuley (1985a) has observed that as the concentration of K^+ increased relative to Na^+, the number of algae increased in European hydra, but no overgrowth occurred. In our preliminary screening, a K^+/Na^+ ratio of less than 0.4 resulted in a modest increase in number of algae per cell, but only after 8 days of treatment. The trend is opposite to that reported by McAuley (1985a) for European hydra.

Douglas and Smith (1984) hypothesize that number of algae per cell in green hydra is controlled by intracellular pH. They argue that since symbiotic *Chlorella* release substantial amounts of extracellular carbon as maltose at low pH (Muscatine, 1965; Mews, 1980), much less carbon is available for synthesis of new algal cell material. But if perialgal vacuolar pH were to increase temporarily, maltose release would be slight, leaving more carbon available for growth of new cells, especially at higher irradiances. The effect on population density would be exacerbated if, at the same time, the growth of the host cells was diminished by starvation. The effect of inorganic nutrients on ultracellular pH, as well as a specific ion effect, awaits support from direct evidence.

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